

# The *MRP4/ABCC4* Gene Encodes a Novel Apical Organic Anion Transporter in Human Kidney Proximal Tubules: Putative Efflux Pump for Urinary cAMP and cGMP

RÉMON A. M. H. VAN AUBEL,\* PASCAL H. E. SMEETS,\* JANNY G. P. PETERS,\* RENÉ J. M. BINDELS,<sup>†</sup> and FRANS G. M. RUSSEL\*

*Departments of \*Pharmacology and Toxicology and <sup>†</sup>Cell Physiology, Nijmegen Center for Molecular Life Sciences, Nijmegen, The Netherlands.*

**Abstract.** The cyclic nucleotides cAMP and cGMP play key roles in cellular signaling and the extracellular regulation of fluid balance. In the kidney, cAMP is excreted across the apical proximal tubular membrane into urine, where it reduces phosphate reabsorption through a dipyridamole-sensitive mechanism that is not fully understood. It has long been known that this cAMP efflux pathway is dependent on ATP and is inhibited by probenecid. However, its identity and whether cGMP shares the same transporter have not been established. Here the expression, localization, and functional properties of human multidrug resistance protein 4 (MRP4) are reported. MRP4 is localized to the proximal tubule apical membrane of human kidney, and membrane vesicles from Sf9 cells expressing human MRP4 exhibit ATP-

dependent transport of [<sup>3</sup>H]cAMP and [<sup>3</sup>H]cGMP. Both probenecid and dipyridamole are potent MRP4 inhibitors. ATP-dependent [<sup>3</sup>H]methotrexate and [<sup>3</sup>H]estradiol-17 $\beta$ -D-glucuronide transport by MRP4 and interactions with the anionic conjugates *S*-(2,4-dinitrophenyl)-glutathione, *N*-acetyl-(2,4-dinitrophenyl)-cysteine,  $\alpha$ -naphthyl- $\beta$ -D-glucuronide, and *p*-nitrophenyl- $\beta$ -D-glucuronide are also demonstrated. In kidneys of rats deficient in the apical anionic conjugate efflux pump Mrp2, Mrp4 expression is maintained at the same level. It is concluded that MRP4 is a novel apical organic anion transporter and the putative efflux pump for cAMP and cGMP in human kidney proximal tubules.

cAMP and cGMP are second messengers involved in the early response to extracellular signals in virtually every living cell. In many epithelial cells, they also play important roles in the extracellular regulation of fluid balance. It has been known for almost 30 yr that cAMP and cGMP are excreted from the kidney into urine (1). The main source of urinary cAMP seems to be kidney proximal tubules. Stimulation by parathyroid hormone results in elevation of intracellular cAMP levels and subsequent excretion of cAMP into the tubular lumen, where it is converted to adenosine (2). Reuptake of adenosine into proximal tubule cells reduces phosphate reabsorption via an unknown mechanism (3). The relevance of this extracellular cAMP-adenosine pathway has been indicated by the finding that dipyridamole, which inhibits adenosine uptake, resolves urinary phosphate leakage among patients with primary hyperparathyroidism or hypophosphatemia (3,4). Urinary cGMP, which originates from glomeruli under the control of atrial natriuretic peptide (ANP) or nitric oxide, inhibits reabsorption

of sodium and chloride in the distal tubules (5,6). Proximal tubules seem to lack ANP receptors; however, apical proximal tubule membrane vesicles from ANP-infused rat kidneys exhibit decreased sodium uptake (7). Also, ANP inhibits sodium reabsorption in the proximal tubule-derived cell line LLC-PK<sub>1</sub>, which can be mimicked by exogenous cGMP (8). The identity of the proximal tubule urinary excretion route for cAMP and cGMP has not yet been resolved.

Monolayers of LLC-PK<sub>1</sub> cells release both cAMP and cGMP preferentially across the apical membrane, via a probenecid-sensitive, temperature-sensitive, energy-dependent transport mechanism (8,9). Such a profile matches a member of the multidrug resistance protein (MRP/ABCC) subgroup of the ATP-binding cassette (ABC) protein superfamily (10,11). Mrp2 (ABCC2) functions as an ATP-dependent anionic conjugate efflux pump in the apical membrane of renal proximal tubules, small intestinal villi, and hepatocytes (12). The substrate specificity of MRP2, including leukotriene C<sub>4</sub> (LTC<sub>4</sub>), *S*-(2,4-dinitrophenyl)-glutathione (DNP-SG), and estradiol-17 $\beta$ -D-glucuronide (E<sub>2</sub>17 $\beta$ G), exhibits considerable overlap with that of MRP1, which does not interact with cAMP (12,13). Furthermore, rats deficient in functional Mrp2 (Eisai hyperbilirubinemic Spague-Dawley [EHBR]/transport-deficient Wistar[TR<sup>-</sup>]rats) do not display a clear defect in the renal excretion of several organic anions, suggesting the presence of a compensating apical organic anion transporter in the kidney (14). MRP5 (ABCC5) has been identified as a probenecid-

Received September 24, 2001. Accepted November 14, 2001.

Correspondence to Dr. Frans G. M. Russel, Department of Pharmacology and Toxicology 233, Nijmegen Center for Molecular Life Sciences, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands. Phone: +31-24-3613691/3616892; Fax: +31-24-3614214; E-mail: F.Russel@farm.kun.nl

1046-6673/1303-0595

Journal of the American Society of Nephrology

Copyright © 2002 by the American Society of Nephrology

sensitive, ATP-dependent transporter for cGMP and cAMP but is localized to the basolateral membrane upon expression in MDCKII cells (15,16). MRP4 (ABCC4) exhibits the closest resemblance to MRP5, on the basis of structural characteristics, and both proteins mediate resistance to several xenobiotic nucleosides, such as the antiviral therapeutic agent 9-(2-phosphonylmethoxyethyl)adenine (adefovir) (16–18). Pharmacokinetic studies with human subjects have indicated that the kidney is the main excretory pathway for adefovir and cidofovir [(*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine] (19). Furthermore, the excretory pathway is probenecid-sensitive and rate-limiting, which explains the nephrotoxic side effects observed with these drugs (19).

In this report, we provide evidence that MRP4 is a novel renal proximal tubule apical organic anion efflux pump for cAMP and cGMP. We demonstrate the expression and immunolocalization of MRP4 in human kidney and the substrate specificity of recombinant human MRP4 by functional expression.

## Materials and Methods

### Materials

<sup>3</sup>H-labeled methotrexate (MTX) (15 Ci/mmol), [<sup>3</sup>H]cGMP (6.8 Ci/mmol), [2,8-<sup>3</sup>H]cAMP (21.9 Ci/mmol), and [<sup>3</sup>H]adefovir [9-(2-phosphonylmethoxyethyl)-[2,8-<sup>3</sup>H]adenine, 25.5 Ci/mmol] were obtained from Moravek Biochemicals (Brea, CA). [14,15,19,20-<sup>3</sup>H]LTC<sub>4</sub> (158 Ci/mmol) and [6,7-<sup>3</sup>H]E<sub>2</sub>17βG (44 Ci/mmol) were obtained from NEN Life Science (Boston, MA). Creatine phosphate and creatine kinase were obtained from Boehringer Mannheim (Almere, The Netherlands). Peptide-*N*-glycosidase F (PNGase F) was obtained from New England Biolabs (Westburg, Leiden, The Netherlands). NC45 and ME25 filters were obtained from Schleicher and Schuell (Dassel, Germany). DNP-SG and *N*-acetyl-(2,4-dinitrophenyl)cysteine (NAc-DNP-Cys) were prepared by using 1-fluoro-2,4-dinitrobenzene as the derivatizing reagent and reduced glutathione (GSH) or *N*-acetylcysteine, respectively, as the thiol (20). All other compounds were obtained from Sigma (Zwijndrecht, The Netherlands). Adefovir and cidofovir were kindly provided by Dr. M. Miller, Gilead Sciences (Foster City, CA).

### Isolation of Full-Length Human MRP4 and Partial Rat MRP4 cDNA

Total RNA was isolated from human kidney cortex by acid guanidinium isothiocyanate-phenol-chloroform extraction and was reverse-transcribed in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 3 mM MgCl<sub>2</sub>, supplemented with 0.5 mM dNTP, 10 μM reverse primer (see below), and 200 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Breda, The Netherlands). For amplification of two overlapping MRP4 cDNA fragments, primers were designed on the basis of the published human sequence (nucleotides 1 to 3979, GenBank accession number AF071202). The forward primer M<sub>4</sub>KK [5'-GGTACCGCCACCAT-GCTGCCCGTG-3']; the *Kpn*I site is underlined, a partial Kozak sequence (21), introduced for future studies, is in bold, and ATG is italic] and the reverse primer M<sub>4</sub>R2 (5'-GGCTCTCCAGAGCAC-CATCTTTCAAGG-3', nucleotides 2003 to 2029) were used to amplify the 5' cDNA fragment. For amplification of the 3' cDNA fragment, the forward primer M<sub>4</sub>F2 (5'-TGGTGCAGAAGGGGA-CTTACTGAGTTC-3', nucleotides 1832 to 1860) and the reverse

primer M<sub>4</sub>R3 (5'-CCTTCGGAACGGACTTGACATTTT-3', nucleotides 3987 to 4010) were used. PCR was performed with the Advantage-2 PCR enzyme system (Clontech, Leusden, The Netherlands), which includes a nuclease-deficient *Taq* DNA polymerase and a proofreading polymerase. Amplifications were performed for 30 cycles of 94°C for 45 s, 55°C for 30 s, and 72°C for 3 min. The PCR fragments obtained were purified and cloned into pGEM-T Easy (Promega, Leiden, The Netherlands). Multiple clones were analyzed by cycle sequencing using an ABI 377 automated sequence (Applied Biosystems, Foster City, CA). The coding sequence was identical to the published sequence (GenBank/EMBL accession number AF071202) except for two 1-bp alterations (guanine for adenine at nucleotide positions 2030 and 3800), which resulted in substitution of arginine for glutamine at position 677 and glycine for serine at position 1267. A full-length human MRP4 cDNA in the *Eco*RI and *Spe*I sites of pGEM-T-Easy (pGTE-MRP4) was obtained by assembling the 5' (nucleotides 1 to 1993) and 3' (nucleotides 1993 to 4010) cDNA fragments.

Total RNA was isolated from rat kidney cortex and reverse-transcribed with 10 μM oligo(dT) as described above. PCR with the human MRP4 primers described by Kool *et al.* (22) was performed for 35 cycles of 94°C for 30 s, 30°C for 1 min, and 72°C for 1 min. The resulting 239-bp fragment was cloned into the *Sma*I site of pBlue-script KS (pBS-Mrp4) and sequenced as described above. The partial rat *Mrp4* cDNA has been submitted to GenBank/EMBL (accession number AF376781).

### Antibodies

The linker region (amino acids 611 to 676) of human MRP4 was chosen as the epitope for a polyclonal antibody (pAb). With the M<sub>4</sub>F2 and M<sub>4</sub>R2 primers, the encoding cDNA was amplified from pGTE-MRP4 and cloned in-frame into the expression vector pGEX-3x (Pharmacia, Woerden, The Netherlands). A specific rat *Mrp4* cDNA, which encodes a protein with 93% identity to amino acids 1165 to 1231 of human MRP4, was amplified from pBS-Mrp4 with the forward primer 5'-TAAAATGGACTGAACTAG-3' and the reverse primer 5'-AATGGTGAGAACAGTGCA-3'. The resulting 202-bp fragment was cloned in-frame into pGEX-2T. Glutathione *S*-transferase (GST) fusion proteins were expressed in DH5α *Escherichia coli* cells and isolated by glutathione-Sepharose 4B (Amersham, Uppsala, Sweden) affinity chromatography. Rabbits were immunized and boosted three times with fusion protein (400 and 200 μg, respectively), followed by collection of serum, which was affinity-purified with a column of immobilized GST and then a column of immobilized GST-fusion protein. The eluted, affinity-purified, anti-rat *Mrp4* (pAb rM4-p1; 2 mg/ml) and anti-human *Mrp4* (pAb hM4-p3; 3 mg/ml) antibodies were used in immunohistochemical and immunoblot analyses (see below). Monoclonal antibody (mAb) M<sub>2</sub>III-6 is directed against rat *Mrp2* and cross-reacts with human MRP2 (23), and pAb G34 is directed against the rat Na<sup>+</sup>/K<sup>+</sup>-ATPase (24). A pAb directed against the rat sodium/phosphate type IIa cotransporter was kindly provided by Dr. J. Biber (Institute of Physiology, University of Zurich, Zurich, Switzerland).

### Immunohistochemical Analyses

Normal tissue from a nephrectomized human kidney was provided by the Department of Pathology; its use for medical study was approved by the Nijmegen University institutional review board. Kidney slices were fixed in 1% (vol/vol) periodate-lysine-paraformaldehyde (PLP) fixative for 2 h, washed with 20% (vol/vol) sucrose in phosphate-buffered saline (PBS), and subsequently frozen in liquid N<sub>2</sub>

(25). Sections were air-dried, pretreated for 5 min at 100°C with citrate buffer (10 mM citric acid, pH 6.0), blocked with 10% (vol/vol) goat serum for 30 min at room temperature, and incubated with pAb rM4-p1 or hM4-p3 and M<sub>2</sub>III-6 for 16 h at 4°C. Pretreatment with citrate buffer enhanced the labeling with pAb rM4-p1 and hM4-p3 and was essential for the use of mAb M<sub>2</sub>III-6 in double-labeling experiments. After being washed with PBS, sections were incubated with Alexa 488-conjugated goat anti-rabbit IgG or Alexa 594-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR) for 1 h at room temperature, for observation of MRP4 and MRP2, respectively. Finally, sections were washed with PBS and methanol, mounted in Mowiol, and analyzed by immunofluorescence microscopy.

### Expression of Human MRP4 in Sf9 Cells

From the construct pGTE-MRP4, an *Eco*RI fragment containing the full-length MRP4 cDNA was cloned into the *Eco*RI site of the pFASTBAC1 multiple cloning site, to create pFB1-MRP4. Recombinant baculovirus encoding human MRP4 was generated by using the Bac-To-Bac baculovirus expression system (Life Technologies), and Sf9 cells were infected as described (26). For control experiments, Sf9 cells were infected with recombinant baculovirus encoding the  $\beta$ -subunit of rat H<sup>+</sup>/K<sup>+</sup>-ATPase (HK $\beta$ ).

### Preparation of Membrane Vesicles from Kidney and Sf9 Cells

Brush border membrane vesicles (BBMV) and basolateral membrane vesicles (BLMV) from rat and human kidney cortex were isolated as described (27). Membrane vesicles from Sf9-MRP4 and control cells were isolated as described (28).

### Deglycosylation Study and Immunoblot Analyses

Membrane vesicles were treated with PNGase F according to the instructions provided by the manufacturer. Immunoblotting was performed as described previously (26). Blots were incubated with pAb rM4-p1 (2  $\mu$ g/ml), pAb hM4-p3 (6  $\mu$ g/ml), G34 antiserum (diluted 1:1000), or M<sub>2</sub>III-6 (diluted 1:10) according to the same protocol. Densitometric analyses were performed by using Image Pro Plus 3.0 (Media Cybernetics, Silver Spring, MD).

### Transport Studies in Membrane Vesicles

Freshly prepared membrane vesicles (150  $\mu$ g protein-equivalents) from Sf9 cells expressing MRP4 or HK $\beta$  were prewarmed at 37°C for 1 min and added to prewarmed transport buffer [10 mM MgCl<sub>2</sub>, 40 mM 3-(*N*-morpholino)propanesulfonic acid-Tris, pH 7.0, 50 mM KCl] supplemented with an ATP-regenerating mixture (4 mM ATP, 10 mM creatine phosphate, and 100  $\mu$ g/ml creatine kinase) and a <sup>3</sup>H-labeled compound (28). At indicated times, samples were withdrawn from the reaction mixture, diluted in cold transport buffer, and filtered through NC45 filters. Samples from [<sup>3</sup>H]adefovir uptake studies were filtered through ME25 filters. After being washed with 5 ml of transport buffer, filters were dissolved in liquid scintillation fluid for determination of bound radioactivity. In control experiments, ATP was replaced by the nonhydrolyzable analog  $\beta$ , $\gamma$ -methylene-ATP (AMP-PCP). Net ATP-dependent transport was calculated by subtracting values measured in the presence of AMP-PCP from those measured in the presence of ATP.

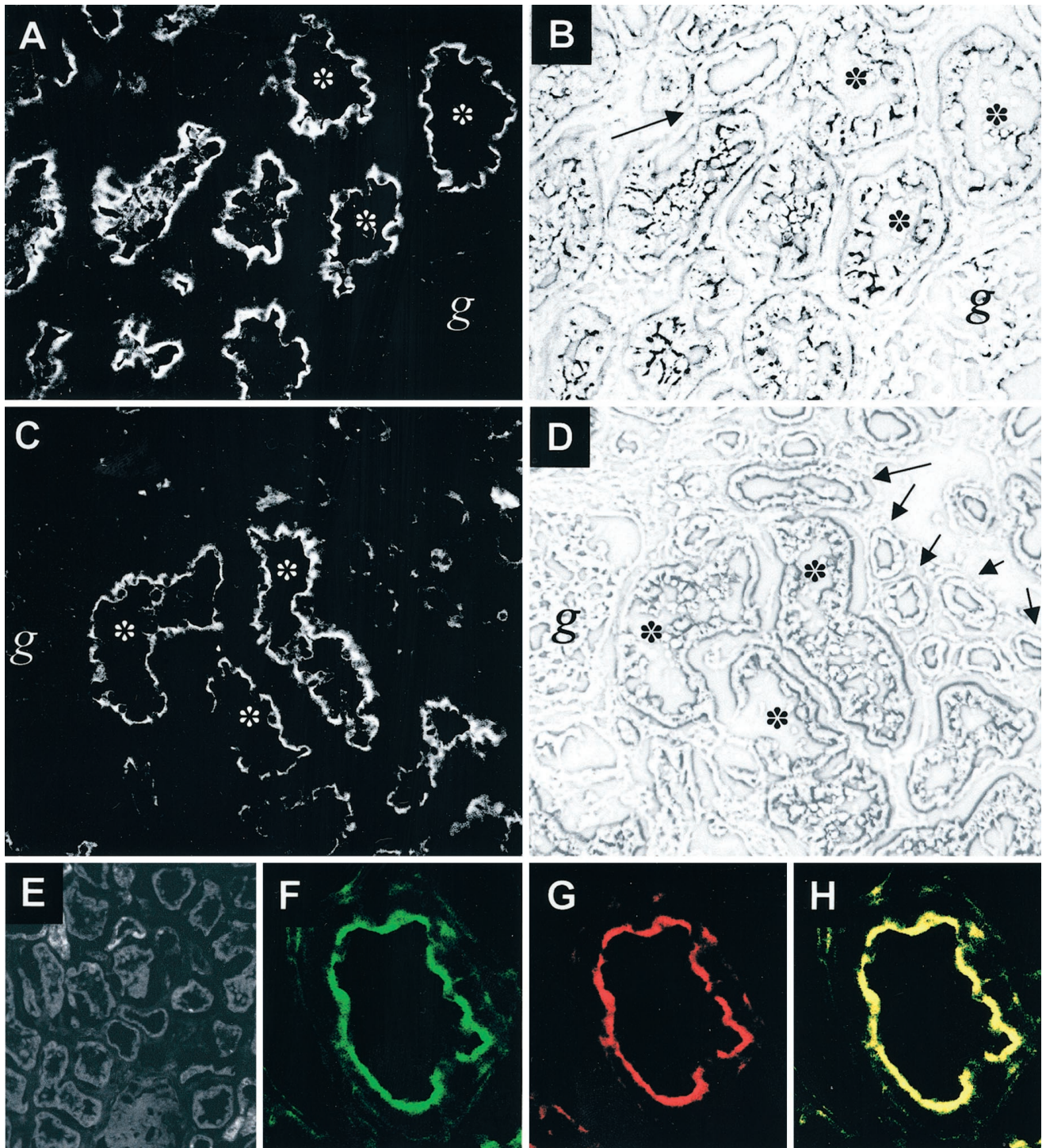
## Results

The localization of MRP4 in kidneys was investigated with single- and double-labeling immunofluorescence microscopic

analyses of PLP-fixed human kidney sections. To this end, pAb were raised against the linker region of human MRP4 (pAb hM4-p3) and a region near the carboxy terminus of rat MrP4 (pAb rM4-p1). Incubation of sections with affinity-purified pAb hM4-p3 demonstrated specific immunofluorescence labeling at the brush border (apical) membrane of proximal tubules (Figure 1, A to D). Identical results were obtained with affinity-purified pAb rM4-p1 on PLP-fixed rat kidney sections (data not shown). The integrity of the proximal tubules with MRP4-specific labeling was confirmed by phase-contrast microscopy (Figure 1, B and D). We observed specific staining of the glomeruli, but the signal intensity varied in different labeling experiments and was lower than that observed for proximal tubule brush border membranes. Distal tubules located in the kidney cortex, which can be recognized on the basis of apical membranes with a characteristic absence of a microvillar structure, were devoid of staining (Figure 1, C and D). When kidney sections were incubated with the secondary antibody alone (Figure 1E), with preimmune serum, or with fusion protein-pretreated hM4-p3 antibodies (data not shown), no staining was observed. Double-labeling of human kidney sections with pAb hM4-p3 and mAb M<sub>2</sub>III-6 demonstrated colocalization of MRP4 and MRP2 (Figure 1, F to H).

The expression of human MRP4 and rat Mrp4 in kidneys was further examined by immunoblotting of BBMV and BLMV from human and rat kidneys. Human MRP4 was detected as a single band of 170 kD in the BBMV fraction, using pAb hM4-p3 (Figure 2A). Identical results were obtained for rat Mrp4 by using pAb rM4-p1. To confirm the isolation procedure for membrane vesicle fractions, the expression of MRP2 and the Na<sup>+</sup>/K<sup>+</sup>-ATPase was investigated. As expected, human MRP2 (190 kD) was detected in the BBMV fraction, whereas the  $\beta$ -subunit of human Na<sup>+</sup>/K<sup>+</sup>-ATPase (55 kD) was detected in the BLMV fraction. Identical results were obtained with BBMV and BLMV from rat kidney (data not shown). Both pAb hM4-p3 and rM4-p1 detected recombinant MRP4 expressed in Sf9 cells (Figure 2B), but the size was smaller than that of MRP4 detected in human kidneys. This difference in size can be attributed to the lack of complex glycosylation in Sf9 cells, which was confirmed by treatment of BBMV and membrane vesicles from Sf9-MRP4 cells with PNGase F (Figure 2C). The molecular weight of MRP4 detected in Sf9-MRP4 cells and PNGase F-treated BBMV matches the size calculated for MRP4 cDNA.

To investigate whether MRP4 is a candidate transporter for cAMP and cGMP, uptake studies were performed with membrane vesicles isolated from Sf9-MRP4 and Sf9-HK $\beta$  cells. The uptake of 100  $\mu$ M [<sup>3</sup>H]cAMP (Figure 3A) or 1  $\mu$ M [<sup>3</sup>H]cGMP (Figure 3B) into membrane vesicles from Sf9-MRP4 cells was stimulated in the presence of ATP and was linear up to 12 min of incubation. The initial net MRP4-mediated, ATP-dependent uptake rates for [<sup>3</sup>H]cAMP (Figure 3C) and [<sup>3</sup>H]cGMP (Figure 3D) were 8 pmol/mg per min (eightfold higher than control values) and 78 fmol/mg per min (12-fold higher than control values), respectively. MRP4-mediated, ATP-dependent [<sup>3</sup>H]cGMP transport was inhibited by probenecid (IC<sub>50</sub> < 100  $\mu$ M) and cAMP (IC<sub>50</sub> < 250  $\mu$ M)

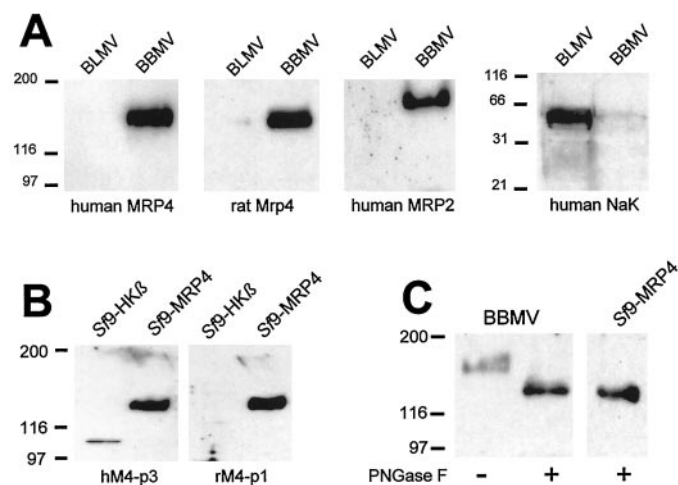


**Figure 1.** Immunofluorescence microscopic analyses of multidrug resistance protein 4 (MRP4) in human kidney. Proximal tubules (\*) surrounding the glomerulus (g) exhibited staining at the brush border membrane with polyclonal antibody (pAb) hM4-p3, directed against human MRP4 (A and C). Distal tubules (arrows) were negative for MRP4 expression. Phase-contrast microscopic analyses of sections identical to A and C are also shown (B and D, respectively). No staining was observed when the secondary antibody was used alone (E). Double-labeling of a human kidney section with pAb hM4-p3 (F) and monoclonal antibody M<sub>2</sub>III-6 (G) was used for detection of MRP4 and MRP2, respectively. Superimposition demonstrates colocalization of MRP4 with MRP2 in the proximal tubule brush border membrane (H).

(Table 1). Dipyridamole proved to be a strong inhibitor of MRP4 (Table 1).

Adefovir and cidofovir are excreted into urine via a probe-

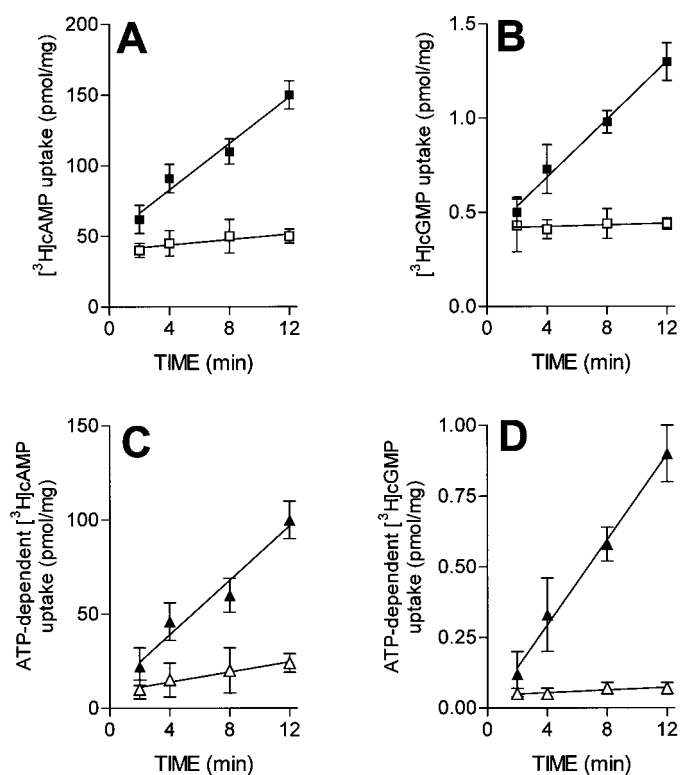
naid-sensitive transport mechanism (19), and MRP4 was recently demonstrated to mediate resistance to adefovir (17,18). However, MRP4-mediated, ATP-dependent [<sup>3</sup>H]cGMP trans-



**Figure 2.** Immunoblot analysis of MRP4 expression in kidney and Sf9-MRP4 cells. (A) MRP4 detected in human kidney cortex brush border membrane vesicles (BBMV) but not basolateral membrane vesicles (BLMV). Identical results were obtained for MRP4 in rat kidney. Expression of human MRP2 and Na<sup>+</sup>/K<sup>+</sup>-ATPase (NaK) was observed in BBMV and BLMV, respectively. (B) pAb hM4-p3 and rM4-p1 detection of recombinant MRP4 expressed in Sf9 cells. (C) Deglycosylation of MRP4 in BBMV from human kidney and Sf9-MRP4 cells. Sizes of protein standards are indicated in kilodaltons. PNGase F, peptide-N-glycosidase F; HK $\beta$ , rat H<sup>+</sup>/K<sup>+</sup>-ATPase  $\beta$ -subunit.

port was inhibited by neither adefovir nor cidofovir, even at concentrations 3 orders of magnitude higher than the substrate concentration (Table 1). Furthermore, uptake of [<sup>3</sup>H]adefovir into membrane vesicles from Sf9-MRP4 cells in the presence of ATP was not different from uptake in the presence of AMP-PCP (data not shown). GSH, glucuronate, or N-acetylcysteine was added as a factor to potentially stimulate MRP4 transport, as previously demonstrated for MRP2-mediated, ATP-dependent [<sup>3</sup>H]vinblastine transport (29). Neither of these conditions resulted in ATP-dependent uptake of [<sup>3</sup>H]adefovir by MRP4.

MTX is eliminated from the body largely via the kidneys, and it is a substrate for MRP2 (28). We investigated whether it is also transported by MRP4. In the presence of ATP, uptake of 0.1  $\mu$ M [<sup>3</sup>H]MTX into Sf9-MRP4 membrane vesicles was linear up to 15 min (Figure 4A). The net ATP-dependent [<sup>3</sup>H]MTX uptake rate was approximately 10-fold higher than that for Sf9-HK $\beta$  membrane vesicles. MRP4-mediated, ATP-dependent [<sup>3</sup>H]MTX uptake increased with increasing MTX concentrations. Fitting of the recorded values to the Michaelis-Menten equation yielded a  $V_{max}$  value of  $430 \pm 30$  pmol/mg per min and a  $K_m$  value of  $1.3 \pm 0.2$  mM (Figure 4B). Concentration-dependent inhibition of ATP-dependent [<sup>3</sup>H]MTX transport by MRP4 was observed for DNP-SG and NAc-DNP-Cys, both with  $IC_{50}$  values of approximately 10  $\mu$ M (Table 2). MRP4 was not inhibited by LTC<sub>4</sub> (Table 2), and Sf9-MRP4 membrane vesicles did not exhibit ATP-dependent uptake of [<sup>3</sup>H]LTC<sub>4</sub> with concentrations of either 5 nM or 1  $\mu$ M. MRP4 was also inhibited by the glucuronide conjugates



**Figure 3.** ATP-dependent transport of [<sup>3</sup>H]cAMP and [<sup>3</sup>H]cGMP by MRP4. Membrane vesicles from Sf9-MRP4 cells were incubated with 100  $\mu$ M [<sup>3</sup>H]cAMP (A) or 1  $\mu$ M [<sup>3</sup>H]cGMP (B), in the presence of ATP (■) or  $\beta$ , $\gamma$ -methylene-ATP (AMP-PCP) (□). Net ATP-dependent uptake of [<sup>3</sup>H]cAMP (C) or [<sup>3</sup>H]cGMP (D) into membrane vesicles from Sf9 cells expressing MRP4 (▲) or HK $\beta$  (△) was determined. Data represent the mean  $\pm$  SEM of three determinations.

$\alpha$ -naphthyl- $\beta$ -D-glucuronide ( $IC_{50} < 100$   $\mu$ M), *p*-nitrophenyl- $\beta$ -D-glucuronide ( $IC_{50} < 1000$   $\mu$ M), and E<sub>2</sub>17 $\beta$ G (Table 2). Sf9-MRP4 membrane vesicles exhibited time- and ATP-dependent uptake of [<sup>3</sup>H]E<sub>2</sub>17 $\beta$ G at a concentration of 500  $\mu$ M (17.6 mCi/mmol), but uptake was slow (Figure 5). At a tracer concentration of 0.2  $\mu$ M, we did not observe MRP4-mediated, ATP-dependent [<sup>3</sup>H]E<sub>2</sub>17 $\beta$ G transport, although, under identical conditions, uptake was observed in membrane vesicles from Sf9 cells expressing human MRP2 (data not shown).

Comparative studies have indicated that the absence of Mrp2 in TR<sup>-</sup> rat kidneys has no significant implications for the renal excretion of several organic anions. For evaluation of the level of expression of Mrp4, the BBMV fractions of kidney cortex from three wild-type rats and three TR<sup>-</sup> rats were isolated and subjected to immunoblot analysis using pAb rM4-p1 (Figure 6). To exclude differences in the yields of BBMV from different rats, protein-equivalent values were normalized to the alkaline phosphatase activity. The level of expression of the apical sodium/phosphate type IIa cotransporter (NaPi-IIa) was included as an internal control. Densitometric analysis indicated that the Mrp4/NaPi-IIa cotransporter ratio in wild-type rats ( $0.64 \pm 0.23$ ) was not significantly different from that in TR<sup>-</sup> rats ( $0.74 \pm 0.18$ ).

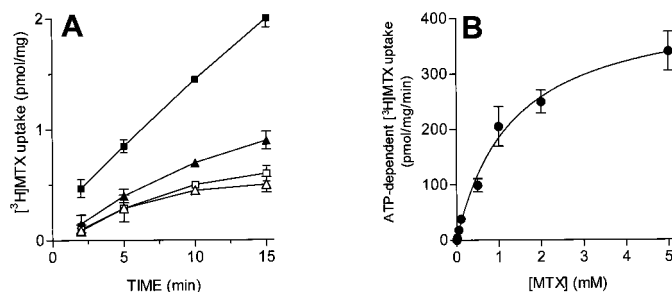
**Table 1.** Effects of probenecid, dipyridamole, and nucleosides on MRP4-mediated, ATP-dependent, [<sup>3</sup>H]cGMP transport<sup>a</sup>

Compound	Concentration (μM)	[ <sup>3</sup> H]cGMP Uptake (% of Control)
No inhibitor		100
Probenecid	10	86 ± 4 <sup>b</sup>
	100	40 ± 3 <sup>c</sup>
	1000	10 ± 3 <sup>c</sup>
cAMP	10	78 ± 26
	50	60 ± 7 <sup>c</sup>
	250	36 ± 9 <sup>c</sup>
Dipyridamole	1	9 ± 3 <sup>c</sup>
Adefovir	1	98 ± 7
	1000	102 ± 9
Cidofovir	1	100 ± 4
	1000	97 ± 12

<sup>a</sup> Membrane vesicles from Sf9-MRP4 cells were incubated with 1 μM [<sup>3</sup>H]cGMP for 10 min at 37°C, in the absence (control) or presence of various compounds. Net ATP-dependent uptake was expressed as a percentage of control uptake. Data represent mean ± SEM values of three determinations. MRP4, multidrug resistance protein 4.

<sup>b</sup> *P* < 0.05.

<sup>c</sup> *P* < 0.01 (ANOVA with Bonferroni's correction).



**Figure 4.** ATP- and concentration-dependent transport of [<sup>3</sup>H]methotrexate ([<sup>3</sup>H]MTX) by MRP4. (A) Time-dependent uptake of [<sup>3</sup>H]MTX (0.1 μM) into membrane vesicles from Sf9-MRP4 (squares) and Sf9-HKβ (triangles) cells at 37°C. Solid and open symbols represent uptake in the presence of ATP and AMP-PCP, respectively. (B) Incubation of Sf9-MRP4 membrane vesicles for 10 min at 37°C in the presence of [<sup>3</sup>H]MTX concentrations ranging from 0.1 μM to 5 mM. Net ATP-dependent uptake values were fitted according to the Michaelis-Menten equation. Data represent the mean ± SEM of three determinations.

## Discussion

The release of cAMP and cGMP in urine is an important step in their functioning as paracrine modulators of renal fluid homeostasis. It has been suggested that these endogenous nucleotides reach the tubular fluid via an ATP-dependent organic anion transporter (3). However, the identity of this transporter has not been established, and it is not known whether cAMP and cGMP share the same export pathway. In this report, we clearly demonstrate, using immunohistochemical

**Table 2.** Effects of glucuronide and glutathione conjugates on MRP4-mediated, ATP-dependent, [<sup>3</sup>H]MTX transport<sup>a</sup>

Compound	Concentration (μM)	[ <sup>3</sup> H]MTX Uptake (% of Control)
No inhibitor		100
DNP-SG	1	86 ± 7 <sup>b</sup>
	10	57 ± 8 <sup>c</sup>
	100	3 ± 4 <sup>c</sup>
NAc-DNP-Cys	1	61 ± 4 <sup>c</sup>
	10	47 ± 8 <sup>c</sup>
	100	29 ± 3 <sup>c</sup>
Leukotriene C <sub>4</sub>	1	102 ± 4
α-Naphthyl-β-D-glucuronide	10	75 ± 2 <sup>c</sup>
	100	25 ± 3 <sup>c</sup>
	1000	18 ± 1 <sup>c</sup>
<i>p</i> -Nitrophenyl-β-D-glucuronide	10	88 ± 4 <sup>b</sup>
	100	75 ± 2 <sup>c</sup>
	1000	25 ± 5 <sup>c</sup>
Estradiol-17β-D-glucuronide	100	75 ± 2 <sup>c</sup>

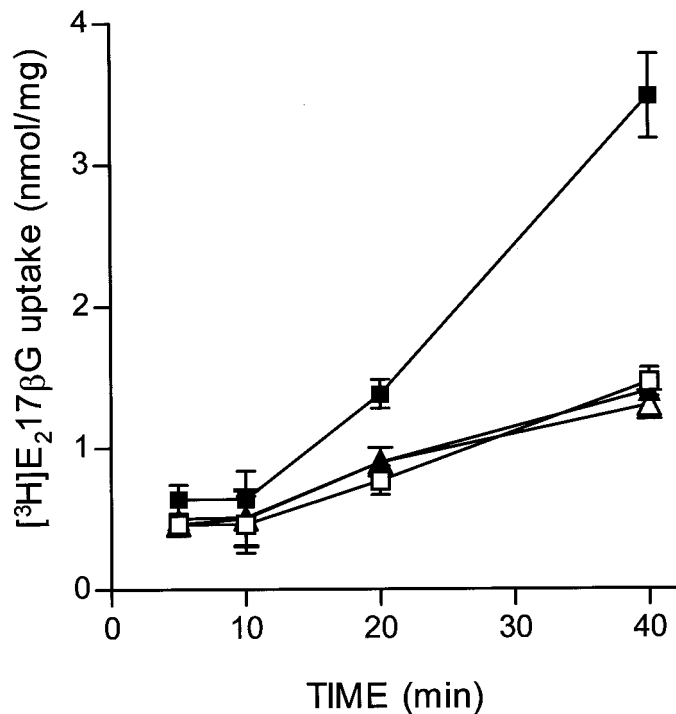
<sup>a</sup> Membrane vesicles from Sf9-MRP4 cells were incubated with 0.1 μM [<sup>3</sup>H]MTX for 10 min at 37°C, in the absence (control) or presence of various compounds. Net ATP-dependent uptake was expressed as a percentage of control uptake. Data represent mean ± SEM values of three to six determinations. MTX, methotrexate; DNP-SG, *S*-(2,4-dinitrophenyl)-glutathione; NAc-DNP-Cys, *N*-acetyl (2,4,-dinitrophenyl)-cysteine.

<sup>b</sup> *P* < 0.05.

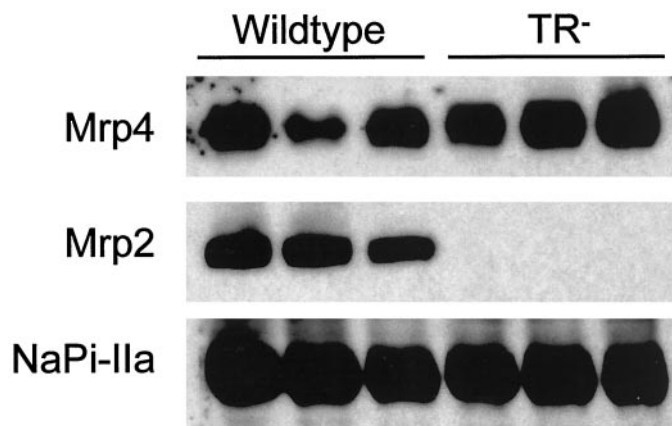
<sup>c</sup> *P* < 0.01 (ANOVA with Bonferroni's correction).

and immunoblot analyses, that MRP4 is expressed at the apical (brush border) membrane of human kidney proximal tubules and that MRP4 mediates ATP-dependent transport of [<sup>3</sup>H]cAMP and [<sup>3</sup>H]cGMP. Furthermore, we demonstrate the interaction of MRP4 with various anionic conjugates and MTX.

The localization of MRP4 and its ability to transport cAMP are in agreement with the results of previous functional studies that indicated the release of cAMP from renal proximal tubule cells upon stimulation by parathyroid hormone, leading to intracellular cAMP levels of at least 100 μM (9). Urinary cAMP has been suggested to originate from plasma cAMP by uptake via basolateral organic anion transporter 1 (OAT1) (1,30). However, plasma cAMP concentrations are in the nanomolar range (31), which may be too low for transport by OAT1; furthermore, infused [<sup>3</sup>H]cAMP is almost completely filtered by the glomeruli (1). Excreted cAMP has been demonstrated to be directly involved in the inhibition of phosphate reabsorption, after degradation to adenosine and subsequent uptake into the proximal tubules (3). Dipyridamole inhibits adenosine uptake and can be used to protect patients from renal phosphate leakage (4). Our results indicate that dipyridamole may have dual effects, *i.e.*, reduction of adenosine uptake and also inhibition of cAMP release via MRP4, reducing urinary adenosine availability. The source of urinary cGMP has been localized primarily to the glomeruli (6). However, modulation



**Figure 5.** ATP-dependent transport of [<sup>3</sup>H]estradiol-17β-D-glucuronide ([<sup>3</sup>H]E<sub>2</sub>17βG) by MRP4. Time-dependent uptake of [<sup>3</sup>H]E<sub>2</sub>17βG (500 μM) into membrane vesicles from Sf9 cells expressing MRP4 (squares) or HKβ (triangles) was assessed at 37°C. Solid and open symbols represent uptake in the presence of ATP and AMP-PCP, respectively. Data represent the mean ± SEM of three determinations.



**Figure 6.** Comparison of MRP4 expression in kidneys from wild-type and Mrp2-deficient (TR<sup>-</sup>) rats. BBMVs were isolated from kidneys from three wild-type and three TR<sup>-</sup> rats. Protein-equivalent values were normalized to the alkaline phosphatase activity, and samples were subjected to immunoblot analysis for detection of rat Mrp4, Mrp2, and sodium/phosphate type IIa cotransporter (NaPi-IIa).

of sodium reabsorption by cGMP in proximal tubules has also been reported, as well as the role of extracellular cGMP as a modulator of sodium transport in LLC-PK<sub>1</sub> cells (7,8). The presence of MRP4 in proximal tubules, combined with the

observed substrate specificity, suggests that this part of the nephron also contributes to urinary cGMP. Whereas a high but physiologic concentration of [<sup>3</sup>H]cAMP (100 μM) was used to demonstrate transport by MRP4, a much lower concentration of [<sup>3</sup>H]cGMP (1 μM) was required. These results are in line with previous observations indicating that intracellular levels of cGMP are approximately 20-fold lower than cAMP levels and that efflux of cGMP from cells is significantly more efficient, compared with that of cAMP (31,32). High concentrations of cAMP were needed to inhibit ATP-dependent [<sup>3</sup>H]cGMP transport by MRP4 (this study), similar to findings for MRP5 (although with a lower IC<sub>50</sub> value) (15). During the final preparation of this manuscript, a study by Chen *et al.* (33), in which these cyclic nucleotides were identified as MRP4 substrates, was reported. Further studies with polarized monolayers of proximal tubule cells will be required to confirm that MRP4 is the renal proximal tubule efflux pump for cAMP and cGMP.

MRP4 and MRP5 have recently been associated with cellular resistance against adefovir (17,18). Furthermore, adefovir and cidofovir accumulate in proximal tubule cells, causing nephrotoxicity (19). The proximal tubule basolateral uptake carrier OAT1 was recently demonstrated to transport both adefovir and cidofovir (34). Pharmacologic studies with human subjects have suggested that the apical efflux pump, rather than the uptake carrier, represents the probenecid-sensitive, rate-limiting step in the urinary excretion of these compounds (19). Those data prompted us to speculate that MRP4 is the renal efflux pump for adefovir and cidofovir. However, those compounds did not inhibit MRP4-mediated transport, and we did not observe any evidence of ATP-dependent uptake of [<sup>3</sup>H]adefovir in membrane vesicles from MRP4-expressing Sf9 cells. It therefore remains unclear how MRP4 extrudes xenobiotic monophosphate nucleosides from the cell. Adefovir and cidofovir are monophosphates and are metabolized into di- and triphosphate (the active form) derivatives (35). HPLC analysis has suggested that MRP4 transports only the unmetabolized derivative and that both adefovir and cidofovir are excreted unchanged into the urine (17,19). However, the possibility that the di- or triphosphate metabolite is the actual substrate, which may be difficult to detect because of rapid degradation by extracellular enzymes, cannot be excluded. In the case of cidofovir, a cyclic metabolite has been identified in urine (36). With MRP4 being an efflux transporter for endogenous cyclic nucleosides, the cyclic metabolite, rather than cidofovir itself, may be transported. However, MRP4 and MRP5 may require a cofactor that is as yet unknown. In a similar manner, both MRP1 and MRP2 require GSH for ATP-dependent transport of the anticancer agents vincristine, vinblastine, and daunorubicin (29,37). Our results at least do not support a role for GSH in stimulating drug transport by MRP4.

Rats deficient in functional Mrp2 do not display clear differences in the renal excretion of many organic anions, compared with wild-type rats (14). Furthermore, transport studies with killifish renal proximal tubules have provided evidence for organic anion transporters in addition to MRP2 (38,39). We demonstrate that the substrate specificity of MRP4 overlaps

with that of MRP2. The MRP2 substrates E<sub>2</sub>17βG and MTX are both transported by MRP4, and the affinity of MRP4 for MTX ( $K_m$  of approximately 1 mM) is in the same range as that documented for MRP2 (28). However, E<sub>2</sub>17βG, a high-affinity MRP2 substrate ( $K_m$  of approximately 7 μM), seems to be a low-affinity substrate for MRP4, because a concentration of 500 μM was required for detection of [<sup>3</sup>H]E<sub>2</sub>17βG transport by MRP4 and 100 μM E<sub>2</sub>17βG inhibited MRP4-mediated [<sup>3</sup>H]MTX transport by only 25%. On the basis of the results of inhibitor studies, we propose that α-naphthyl-β-D-glucuronide, *p*-nitrophenyl-β-D-glucuronide, DNP-SG, and NAc-DNP-Cys are substrates for MRP4. Of interest, the excretion of α-naphthyl-β-D-glucuronide from TR<sup>-</sup> rat kidneys was found not to be different from that from wild-type kidneys (40). In rat kidneys, three additional organic anion transporters, *i.e.*, organic anion transporter polypeptide 1 (Oatp1), kidney-specific organic anion-transporter 1 (Oat-k1), and sodium/phosphate cotransporter type 2 (Npt1), are expressed at the proximal tubule brush border membrane, with substrate specificities overlapping those of MRP2 and MRP4 (14,41). A human ortholog has been identified for rat Npt1; however, human counterparts of rat Oatp1 and Oat-k1 do not seem to exist (41,42). Whether rat Mrp4 in TR<sup>-</sup> rats and human MRP4 in patients with Dubin-Johnson syndrome (both lacking functional MRP2) (12) can represent an alternative pathway for the renal excretion of organic anions remains to be established and may even involve differences between species.

## References

- Broadus AE, Kaminsky NI, Hardman JG, Sutherland EW, Liddle GW: Kinetic parameters and renal clearances of plasma adenosine 3',5'-monophosphate and guanosine 3',5'-monophosphate in man. *J Clin Invest* 49: 2222–2236, 1970
- Jackson EK, Dubey RK: Role of the extracellular cAMP-adenosine pathway in renal physiology. *Am J Physiol* 281: F597–F612, 2001
- Friedlander G, Prie D, Siegfried G, Amiel C: Role of renal handling of extracellular nucleotides in modulation of phosphate transport. *Kidney Int* 49: 1019–1022, 1996
- Prie D, Blanchet FB, Essig M, Jourdain JP, Friedlander G: Dipyridamole decreases renal phosphate leak and augments serum phosphorus in patients with low renal phosphate threshold. *J Am Soc Nephrol* 9: 1264–1269, 1998
- Friedlander G, Amiel C: Extracellular nucleotides as modulators of renal tubular transport. *Kidney Int* 47: 1500–1506, 1995
- Huang CL, Ives HE, Cogan MG: *In vivo* evidence that cGMP is the second messenger for atrial natriuretic factor. *Proc Natl Acad Sci USA* 83: 8015–8018, 1986
- Hammond TG, Yusufi AN, Knox FG, Dousa TP: Administration of atrial natriuretic factor inhibits sodium-coupled transport in proximal tubules. *J Clin Invest* 75: 1983–1989, 1985
- Chevalier RL, Fang GD, Garmey M: Extracellular cGMP inhibits transepithelial sodium transport by LLC-PK1 renal tubular cells. *Am J Physiol* 270: F283–F288, 1996
- Strewler GJ: Release of cAMP from a renal epithelial cell line. *Am J Physiol* 246: C224–C230, 1984
- Borst P, Evers R, Kool M, Wijnholds J: A family of drug transporters: The multidrug resistance-associated proteins. *J Natl Cancer Inst* 92: 1295–1302, 2000
- Dean M, Rzhetsky A, Allikmets R: The human ATP-binding cassette (ABC) transporter superfamily. *Genome Res* 11: 1156–1166, 2001
- König J, Nies AT, Cui Y, Leier I, Keppler D: Conjugate export pumps of the multidrug resistance protein (MRP) family: Localization, substrate specificity, and MRP2-mediated drug resistance. *Biochim Biophys Acta* 1461: 377–394, 1999
- Evers R, Cnubben NHP, Wijnholds J, van Deemter L, van Bladeren PJ, Borst P: Transport of glutathione prostaglandin A conjugates by the multidrug resistance protein 1. *FEBS Lett* 419: 112–116, 1997
- van Auel RAMH, Masereeuw R, Russel FGM: Molecular pharmacology of renal organic anion transporters. *Am J Physiol* 279: F216–F232, 2000
- Jedlitschky G, Burchell B, Keppler D: The multidrug resistance protein 5 functions as an ATP-dependent export pump for cyclic nucleotides. *J Biol Chem* 275: 30069–30074, 2000
- Wijnholds J, Mol CA, van Deemter L, de Haas M, Scheffer GL, Baas F, Beijnen JH, Scheper RJ, Hatse S, De Clercq E, Balzarini J, Borst P: Multidrug-resistance protein 5 is a multispecific organic anion transporter able to transport nucleotide analogs. *Proc Natl Acad Sci USA* 97: 7476–7481, 2000
- Schuetz JD, Connelly MC, Sun D, Paibir SG, Flynn PM, Srinivas RV, Kumar A, Fridland A: MRP4: A previously unidentified factor in resistance to nucleoside-based antiviral drugs. *Nature Med* 5: 1048–1051, 1999
- Lee K, Klein-Szanto AJ, Kruh GD: Analysis of the MRP4 drug resistance profile in transfected NIH3T3 cells. *J Natl Cancer Inst* 92: 1934–1940, 2000
- Cundy KC: Clinical pharmacokinetics of the antiviral nucleotide analogues didanosine and zalcitabine. *Clin Pharmacokinet* 36: 127–143, 1999
- Hinchman CA, Matsumoto H, Simmons TW, Ballatori N: Intrahepatic conversion of a glutathione conjugate to its mercapturic acid: Metabolism of 1-chloro-2,4-dinitrobenzene in isolated perfused rat and guinea pig livers. *J Biol Chem* 266: 22179–22185, 1991
- Kozak M: An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res* 15: 8125–8148, 1987
- Kool M, de Haas M, Scheffer GL, Scheper RJ, van Eijk MJT, Juijn JA, Baas F, Borst P: Analysis of expression of *cMOAT* (*MRP2*), *MRP3*, *MRP4*, and *MRP5*, homologues of the multidrug resistance-associated protein gene (*MRP1*) in human cancer cell lines. *Cancer Res* 57: 3537–3547, 1997
- Scheffer GL, Kool M, Heijn M, de Haas M, Pijnenborg AC, Wijnholds J, van Helvoort A, de Jong MC, Hooijberg JH, Mol CA, van der Linden M, de Vree JM, van der Valk P, Oude Elferink RPJ, Borst P, Scheper RJ: Specific detection of multidrug resistance proteins MRP1, MRP2, MRP3, MRP5, and MDR3 P-glycoprotein with a panel of monoclonal antibodies. *Cancer Res* 60: 5269–5277, 2000
- Peters WH, Ederveen AG, Salden MH, De Pont JHHM, Bonting SL: Lack of immunological cross reactivity between the transport enzymes (Na<sup>+</sup>/K<sup>+</sup>)-ATPase and (K<sup>+</sup>/H<sup>+</sup>)-ATPase. *J Bioenerg Biomembr* 16: 223–232, 1984
- Hoenderop JG, Hartog A, Stuver M, Doucet A, Willems PH, Bindels RJM: Localization of the epithelial Ca<sup>2+</sup> channel in rabbit kidney and intestine. *J Am Soc Nephrol* 11: 1171–1178, 2000
- van Auel RAMH, van Kuijk MA, Koenderink JB, Deen PMT, van Os CH, Russel FGM: Adenosine triphosphate-dependent



- transport of anionic conjugates by the rabbit multidrug resistance-associated protein MRP2 expressed in insect cells. *Mol Pharmacol* 53: 1062–1067, 1998
27. Russel FGM, van der Linden PE, Vermeulen WG, Heijn M, van Os CH, van Ginneken CA: Na<sup>+</sup> and H<sup>+</sup> gradient-dependent transport of *p*-aminohippurate in membrane vesicles from dog kidney cortex. *Biochem Pharmacol* 37: 2639–2649, 1988
  28. Bakos E, Evers R, Sinko E, Varadi A, Borst P, Sarkadi B: Interactions of the human multidrug resistance proteins MRP1 and MRP2 with organic anions. *Mol Pharmacol* 57: 760–768, 2000
  29. van Aubel RAMH, Koenderink JB, Peters JGP, van Os CH, Russel FGM: Mechanisms and interaction of vinblastine and reduced glutathione transport in membrane vesicles by the rabbit multidrug resistance protein MRP2 expressed in insect cells. *Mol Pharmacol* 56: 714–719, 1999
  30. Sekine T, Watanabe N, Hosoyamada M, Kanai Y, Endou H: Expression cloning and characterization of a novel multispecific organic anion transporter. *J Biol Chem* 272: 18526–18529, 1997
  31. Steiner AL, Pagliara AS, Chase LR, Kipnis DM: Radioimmunoassay for cyclic nucleotides. II. Adenosine 3',5'-monophosphate and guanosine 3',5'-monophosphate in mammalian tissues and body fluids. *J Biol Chem* 247: 1114–1120, 1972
  32. Geary CA, Goy MF, Boucher RC: Synthesis and vectorial export of cGMP in airway epithelium: Expression of soluble and CNP-specific guanylate cyclases. *Am J Physiol* 265: L598–L605, 1993
  33. Chen ZS, Lee K, Kruh GD: Transport of cyclic nucleotides and estradiol-17- $\beta$ -D-glucuronide by multidrug resistance protein 4: Resistance to 6-mercaptopurine and 6-thioguanine. *J Biol Chem* 276: 33747–33754, 2001
  34. Cihlar T, Lin DC, Pritchard JB, Fuller MD, Mendel DB, Sweet DH: The antiviral nucleotide analogs cidofovir and adefovir are novel substrates for human and rat renal organic anion transporter 1. *Mol Pharmacol* 56: 570–580, 1999
  35. Balzarini J, Hao Z, Herdewijn P, Johns DG, De Clercq E: Intracellular metabolism and mechanism of anti-retrovirus action of 9-(2-phosphonylmethoxyethyl)adenine, a potent anti-human immunodeficiency virus compound. *Proc Natl Acad Sci USA* 88: 1499–1503, 1991
  36. Cundy KC, Bidgood AM, Lynch G, Shaw JP, Griffin L, Lee WA: Pharmacokinetics, bioavailability, metabolism, and tissue distribution of cidofovir (HPMPC) and cyclic HPMPC in rats. *Drug Metab Dispos* 24: 745–752, 1996
  37. Loe DW, Deeley RG, Cole SPC: Characterization of vincristine transport by the  $M_r$  190,000 multidrug resistance protein (MRP): Evidence for cotransport with reduced glutathione. *Cancer Res* 58: 5130–5136, 1998
  38. Masereeuw R, Russel FGM, Miller DS: Multiple pathways of organic anion secretion in renal proximal tubule revealed by confocal microscopy. *Am J Physiol* 271: F1173–F1182, 1996
  39. Masereeuw R, Moons MM, Toomey BH, Russel FGM, Miller DS: Active lucifer yellow secretion in renal proximal tubule: Evidence for organic anion transport system crossover. *J Pharmacol Exp Ther* 289: 1104–1111, 1999
  40. de Vries MH, Redegeld FA, Koster AS, Noordhoek J, de Haan JG, Oude Elferink RPJ, Jansen PLM: Hepatic, intestinal and renal transport of 1-naphthol- $\beta$ -D-glucuronide in mutant rats with hereditary-conjugated hyperbilirubinemia. *Naunyn-Schmiedeberg Arch Pharmacol* 340: 588–592, 1989
  41. Uchino H, Tamai I, Yamashita K, Minemoto Y, Sai Y, Yabuuchi H, Miyamoto K, Takeda E, Tsuji A: *p*-Aminohippuric acid transport at renal apical membrane mediated by human inorganic phosphate transporter NPT1. *Biochem Biophys Res Commun* 270: 254–259, 2000
  42. Kullak-Ublick GA, Ismail MG, Stieger B, Landmann L, Huber R, Pizzagalli F, Fattinger K, Meier PJ, Hagenbuch B: Organic anion-transporting polypeptide B (OATP-B) and its functional comparison with three other OATPs of human liver. *Gastroenterology* 120: 525–533, 2001